

CERCOPITHECUS AETHIOPS KIDNEY CELLS: CYTO-CHEMICAL AND MORPHOLOGICAL CHARACTERISTICS OF CELLS CULTIVATED IN CHEMICALLY DEFINED MEDIUM COMPARED TO CULTURES SUPPLEMENTED WITH HORSE OR FETAL-CALF SERA^{1, 2}

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ABSTRACT

Cultures (NCTC-designated) of kidney cells of *Cercopithecus aethiops* were grown in a chemically defined medium (NCTC-135) and were then examined morphologically and cytochemically. Comparisons were made with cultures of the same cell line in medium supplemented with either 10% fetal-calf or 10% horse sera. The epitheloid cells of the serum-free cultures demonstrated only a slight difference in enzymatic activity when compared to the cultures supplemented by 10% fetal-calf and 10% horse sera. The use of a particular serum supplement in combination with a chemically defined medium did not appear to determine if a cell culture would be reactive or nonreactive to alkaline or acid phosphatase. Results indicated also that alkaline-phosphatase activity was altered very little by freezing of the cells prior to testing. Variability of alkaline-phosphatase was interpreted as a result of a combination of environmental factors and "age" *in vitro*. The strong activity of acid phosphatase, noted both in the serum-free culture (NCTC-4952) and in the culture (NCTC-6314) supplemented by 10% fetal-calf serum, was regarded as reflection of the number of culture passages *in vitro* and the constitutive nature of the enzyme.

INTRODUCTION

Kidney cells of the African green monkey (*Cercopithecus aethiops*) were originated as a cell line in 1961 (Hopps *et al.*, 1963). This cell line has been used in various virological and cytogenetic investigations (Fernandes and Moorhead, 1965; Love and Fernandes, 1965; Meyer *et al.*, 1962; Rabson *et al.*, 1964; Tomkins and Pye, 1968). However, relatively little is known about its morphological and cytochemical properties following adaption to a chemically defined medium. Experimentally, the use of various chemically defined media for the long-term propagation of mammalian cells *in vitro* is well documented (Bakken *et al.*, 1961; Bryant, 1966; Evans *et al.*, 1956, 1959, 1964; McQuilkin *et al.*, 1957; White and Waymouth, 1954). Cells adapted to growth in a chemically defined medium provide an ideally controlled environment for investigations related to cellular activities of a biochemical or cytochemical nature. Moreover, possible damaging effects attributable to serum supplements may be eliminated by this method (LoGrippe, 1958).

The purpose of the present investigation was to determine semiquantitatively possible similarities and/or differences in the alkaline- and acid-phosphatase activities, *in vitro*, of certain NCTC-designated cell lines of different ages. These cell lines were derived from a cell line of *Cercopithecus aethiops* kidney cells (BS-C1) by employing either different sera supplements or no serum supplement in combination with a chemically defined medium (NCTC-135).

MATERIALS AND METHODS

All four cell lines, designated NCTC (National Center for Tissue Culture) (4952, 6313, 6314, 6315), of the present study originated from cultures of African

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green monkey (*Cercopithecus aethiops*) kidney cells. The original culture was initiated by H. Hopps, Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland.

The cell line from which subline NCTC-4952 was originally derived was supplied by Hopps on August 16, 1961, as BS-C1-25-2. This culture was in the 25th culture passage and was received in medium 199 with 20(v/v)% fetal-calf serum and yeast extract. It was switched immediately to 10(v/v)% horse serum and medium NCTC-135 (Evans *et al.*, 1964) and was designated NCTC-3979. This line was adapted to growth in the serum-free medium, NCTC-135, and then received the designation NCTC-4952.

NCTC-6313 was a subline of cell line NCTC-3979 adapted to growth in 1/16(v/v)% horse serum and medium NCTC-135, and was originally designated NCTC-4951. It was frozen and stored in liquid nitrogen on September 2, 1964, and was recovered September 10, 1968, when it was cultured in medium NCTC-135 supplemented with 10(v/v)% horse serum. It was designated NCTC-6313.

Two other lines were obtained from Hopps on September 9, 1968. These cell lines were in the 89th (BS-C1-89-1) and 604th (BS-C1-604-1) culture passages and received the designations NCTC-6315 and NCTC-6314, respectively. Both were maintained in this study in 10(v/v)% fetal-calf serum with medium NCTC-135.

TABLE I
Summary of BS-C1-89, 604, 25* African green monkey kidney cell lines and
NCTC-designated cell lines†

NCTC cell line	Medium used	Serum supplement used	Total culture passages <i>in vitro</i>	Original cell line and culture
NCTC-4952	NCTC-135	None	305	BS-C1-25-2
NCTC-6314	NCTC-135	10% fetal calf	610	BS-C1-604-1
NCTC-6315	NCTC-135	10% fetal calf	110	BS-C1-89-1
NCTC-6313	NCTC-135	10% horse	60	BS-C1-25-2

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The cell lines were maintained in T₁₅ flask cultures at 37.5°C in the following combinations: NCTC-135 without a serum supplement; NCTC-135/10(v/v)% fetal-calf serum; NCTC-135/10(v/v)% horse serum. Table I represents a summary of the NCTC cell-line derivatives of *Cercopithecus aethiops* kidney cells and their respective media components used in this investigation. The amount of medium in all cell cultures was constant and was renewed at two-to-three-day intervals. The cell cultures were subcultured once a week, with the exception of cell line NCTC-6314, which was subcultured twice weekly because of its marked proliferating activity. Each subculture consisted of 7 ml fresh medium and 1 ml of cell suspension. In subculturing, cells were mopped from the glass surface using previously inserted pieces of perforated cellophane (Evans and Earle, 1947), thus avoiding use of various chelating agents and trypsin. All cell cultures were antibiotic-free.

The cytochemical studies were performed on Leighton-tube cultures containing 9-x-50-mm coverslips on which cell monolayers formed. Each Leighton-tube culture consisted of 1.5 ml of fresh medium and 0.5 ml of cell suspension. Each ml of cell suspension consisted of approximately 100,000 cells. The Leighton-tube cultures were gassed with 10% CO₂ in air, stoppered, and then incubated at 37.5°C for 48 hours. Cytochemical reactions performed on coverslip preparations

of the cell monolayers were all processed during the rapid-growth phase. All cytochemical reactions were performed on one day, at weekly intervals, over a two-month period, employing the reactions described below for alkaline and acid phosphatase.

Alkaline Phosphatase

Coverslips containing the cell monolayers were rinsed in saline solution and fixed in cold (4°C) 10% formalin in methanol (Pearse, 1968) for 5 minutes. Alkaline phosphatase activity was determined using naphthol AS-BI-phosphate as the substrate and Fast Violet B salt as the coupler (Burstone, 1958). Incubation was carried out at room temperature for 45 minutes. Sites of alkaline-phosphatase activity appeared as a brown or brownish-black precipitate within the cytoplasm and in the immediate vicinity of the nucleus. Control slides incubated without the substrate were consistently nonreactive. Methyl green was used as a counterstain (Barka and Anderson, 1963).

Acid Phosphatase

Cell monolayers were rinsed in saline solution and fixed in cold (4°C) neutral formalin for 5 minutes. Activity of acid phosphatase was determined using alpha-naphthyl phosphate as the substrate and Fast Garnet GBC salt as the coupler (Barka and Anderson, 1963). The cells were incubated for 1.5 hr at 37°C. Within the cytoplasm, sites of acid-phosphatase activity were indicated by variable-sized reddish-brown granules. Control slides incubated without the substrate were consistently nonreactive. Methyl green was used as a counterstain.

OBSERVATIONS

Light-microscopic examination of the cell monolayers revealed the individual cells to be morphologically epitheloid. These cells presented a loose-network arrangement or a "whirl" pattern (fig. 1). The individual cells of all cultures were characterized by their round ovoid-shaped nuclei, distinct nuclear membranes, and two to three prominent nucleoli.

EXPLANATION OF FIGURES 1-5

- FIGURE 1. NCTC-6313 culture (10% horse serum). The epitheloid cells may be observed forming a "whirl-like" arrangement. This photomicrograph demonstrates the moderate reaction of alkaline-phosphatase activity. Naphthol AS-BI-phosphate was used as the substrate and Fast Violet B salt as the coupler. Methyl green counterstain, $\times 390$.
- FIGURE 2. NCTC-4952 (serum-free culture). This photomicrograph was chosen as representative of the cells demonstrating negative-to-weak activity for alkaline phosphatase in the serum-free cell culture, NCTC-4952, and in the 10%-fetal-calf-serum-supplemented cell cultures, NCTC-6314, 6315. Naphthol AS-BI-phosphate was used as the substrate and Fast Violet B salt as the coupler. Methyl green counterstain, $\times 1300$.
- FIGURE 3. NCTC-6313 (10% horse serum). Alkaline-phosphatase activity of the 10%-horse-serum-supplemented cell cultures was consistently moderate. The pattern of precipitation was patchy. Nucleus was nonreactive. Naphthol AS-BI-phosphate was used as the substrate and Fast Violet B salt as the coupler. Methyl green counterstain, $\times 1300$.
- FIGURE 4. NCTC-6314 (10% fetal-calf serum). The cell is representative of what was interpreted as a strong reaction for acid-phosphatase activity. The reddish-brown precipitate appeared to be concentrated unilaterally. A few reddish-brown granules were also observed within the cytoplasm. The nucleus was nonreactive. Alpha-naphthyl phosphate was used as the substrate and Fast Garnet GBC salt as coupler. Methyl green counterstain, $\times 2400$.
- FIGURE 5. NCTC-6313 (10% horse serum). This cell is representative of a moderate reaction for acid phosphatase. The concentration of the precipitate demonstrated a unilateral and juxtannuclear position. The variable-sized granules are easily identified in the cells of this culture line. Nucleus was nonreactive. Alpha-naphthyl phosphate was used as the substrate and Fast Garnet GBC salt as coupler. Methyl green counterstain, $\times 2400$.

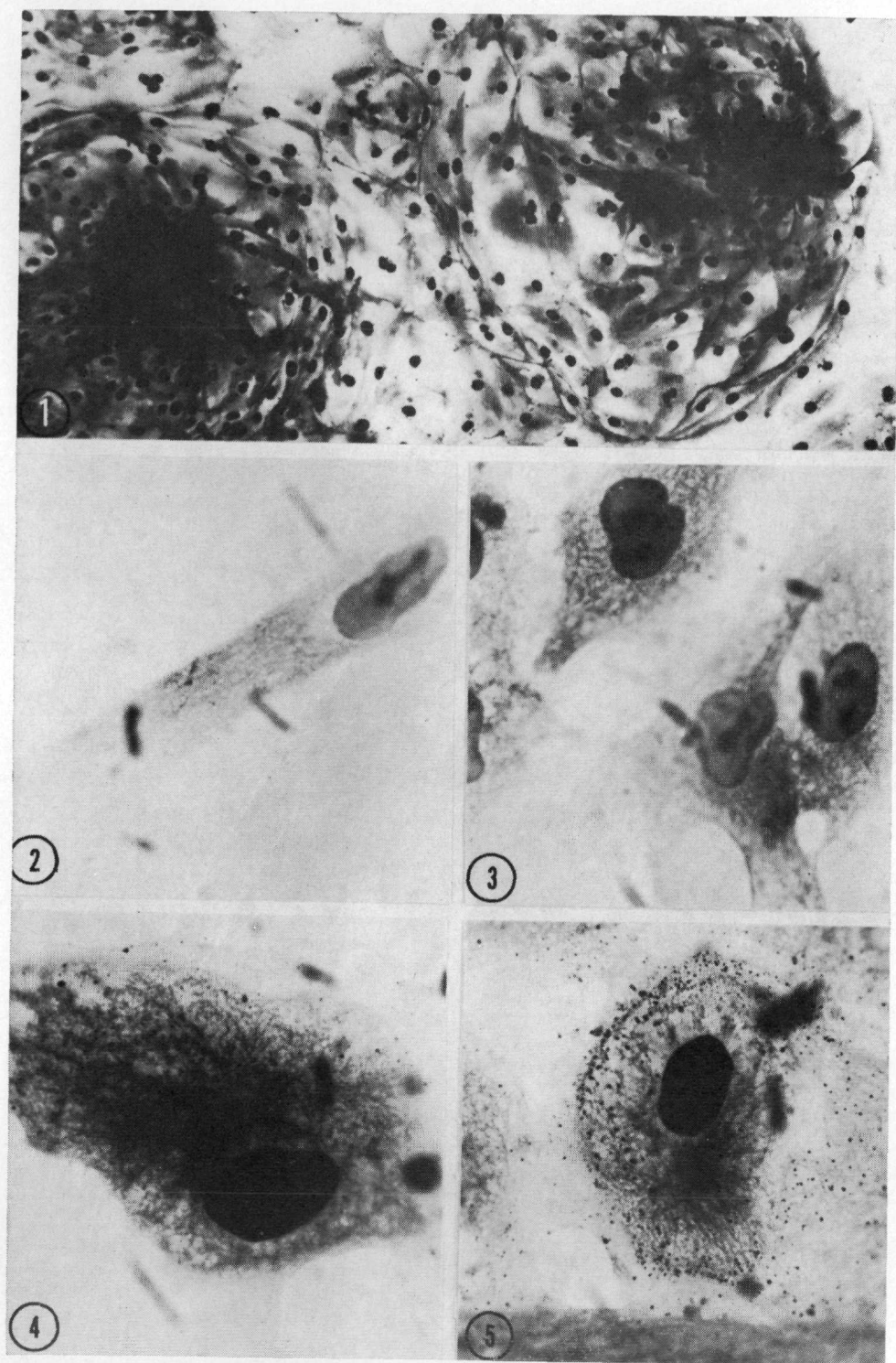


Figure 1-5

Enzymatic activity of the cytochemical reactions was graded on the basis of the intensity of the reaction, employing the following symbols:

- 0 - nonreactive,
 + - weakly reactive,
 ++ - moderately reactive, and
 +++ - strongly reactive.

The grades of activity, listed in table 2, were recorded as an average activity of each NCTC-designated cell culture for the two-month period of observation. Cellular enzyme-activity of the individual cultures was fairly consistent and characteristic for the period of observation.

TABLE 2
Cytochemical properties of NCTC-designated cell cultures of C. aethiops kidney cells

Cell culture	Serum supplement	Alkaline phosphatase	Acid phosphatase
NCTC-4952	None	0+	+++
NCTC-6314	10% fetal calf	0+	+++
NCTC-6315	10% fetal calf	0+	++
NCTC-6313	10% horse	++	++

The epitheloid cells of the serum-free cultures of line NCTC-4952 and the cultures supplemented by 10% fetal-calf serum of lines NCTC-6314 and 6315 were observed to be consistently negative to weak for alkaline-phosphatase activity. The cell shown in figure 2 is typical of the negative-to-weak activity of alkaline phosphatase observed in the above three NCTC-designated cultures. In contrast, the epitheloid cells of line NCTC-6313 (10% horse serum) were observed to be moderate in reaction for alkaline-phosphatase activity (fig. 3). The pattern of precipitation observed presented a patchy distribution within the cytoplasm. The nucleus was nonreactive.

Acid-phosphatase activity of the epitheloid cells of the four NCTC-designated cultures was observed to differ with respect to the number of culture passages *in vitro* rather than with respect to the absence or presence of a particular serum supplement. The latter observation was particularly true in the case of the NCTC-designated cultures 4952 and 6314. The cells of the serum-free culture, NCTC-4952, were consistently strong for acid-phosphatase activity. The acid-phosphatase activity of NCTC-6314 was consistently stronger than was that of NCTC-6315. Both of these lines had received a supplement of 10% fetal-calf serum, but their culture passages *in vitro* were significantly different (table 1). The intracytoplasmic localization of the reaction appeared to be concentrated unilaterally and presented a reddish-brown precipitate (fig. 4). In a number of cells reddish-brown granules of variable size were observed randomly distributed within the cytoplasm (fig. 5). The nucleus was nonreactive. The cells of the 10% horse-serum-supplemented culture, NCTC-6313, were moderate in reaction for acid phosphatase.

DISCUSSION

Cytochemical studies related to the intracellular identification and characterization of enzymatic activity of various cells *in vitro* are well documented (Conklin *et al.*, 1962; Farnes *et al.*, 1968; Fortelius, 1963; Kahn *et al.*, 1962). This is not true, however, regarding the cells of the present study.

In general, the results of the present investigation reveal that epitheloid cells grown in a chemically defined medium (NCTC-135), with or without a serum supplement, are somewhat variable in their activity for the cytochemical reactions performed. Differences in the cellular reactivity of the hydrolytic enzymes, alkaline and acid phosphatase, are more evident when comparisons are made with the cells of the 10% fetal-calf- and 10%-horse-serum-supplemented cultures.

Variability in alkaline-phosphatase activity has been reported for HeLa cells within the same culture (Cox and Macleod, 1961-62), as well as for HeLa cell lines from different laboratories (Fortelius *et al.*, 1960). In the present study, the moderate activity of alkaline phosphatase in the cells of the 10%-horse-serum-supplemented cultures suggests a possible environmental effect attributable to some component of the horse serum. However, results of previous investigators have indicated that the type of serum supplement exerts very little effect upon demonstrable enzymatic activity, particularly that of alkaline phosphatase (Cox and Macleod, 1961-62). Moreover, the type of culture medium apparently does not determine whether a cell line is reactive or nonreactive for alkaline phosphatase, but does appear to influence the degree of cellular activity that can be demonstrated within a positive cell line (Cox and Macleod, 1961-62). Using a chemically defined medium (NCTC-135), Farnes *et al.* (1968) demonstrated that *in vitro* cellular alkaline-phosphatase activity was not influenced by the presence or absence of a particular serum supplement. Yet it has been shown that various serum supplements (Saksela, 1962), including those employed in this study, have produced recognizable chromosomal changes of cells *in vitro* (Mitchell *et al.*, 1969; Parshad and Sanford, 1967, 1968). More specifically, correlation between enzymatic activity of alkaline phosphatase and chromosomal variations *in vitro* has been reported (DeCarli *et al.*, 1963).

The moderate alkaline-phosphatase activity noted in the horse-serum-supplemented cultures of the present study, compared to that in the other NCTC-designated cultures, may be a reflection of differences in culture passages *in vitro*, the "older" cultures reflecting a decrease in activity for the enzyme. Both biochemical and cytochemical studies of various cell lines have indicated such an association (Cristofalo *et al.*, 1967; Fortelius, 1963). Conversely, biochemical determinations of acid-phosphatase activity reveal a slight increase with "aging" of a particular cell line (Cristofalo *et al.*, 1967). The cytochemical studies of the present investigation suggest similar results. The cells of the 10%-horse-serum-supplemented cultures, NCTC-6313, were not in continuous culture, but were frozen for four years and recovered at the time of the present study. Freezing of the cells apparently had little effect on the demonstrable activity of alkaline phosphatase or acid phosphatase. Similar results have been reported by Fortelius (1963). The moderate degree of alkaline-phosphatase activity of these cells suggests the possibility of a higher level of activity reflecting the variability of the enzyme. Perhaps, with continuous culture, cells of NCTC-6313 would approximate the nonreactive-to-weak activity observed for the serum-free and 10%-fetal-calf-serum-supplemented cultures.

The variability of enzymatic activity revealed in the present study may possibly reflect an *in vitro* environmental effect. Whether such observations of increased or decreased activity of the hydrolytic enzymes represent a significant sign of the cell's basic metabolic machinery in the *in vitro* environment remains to be established. Perhaps a correlative study, combining a cytochemical and ultrastructural approach, would contribute to a better understanding and interpretation of the physiological role of these enzymes as they exist within the *in vitro* model system.

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